

A novel "two-in-one" spin trap for direct observation of a substrate-based radical intermediate in the methane monooxygenase reaction cycle

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Methane monooxygenase (MMO) is a nonheme diiron enzyme which catalyzes not only the O₂ dependent oxidation of methane to methanol, but also the hydroxylation/oxidation of all other C₁ to C₈ linear and cyclic hydrocarbon molecules that can gain access to the active site. A number of transient intermediates in the MMO reaction cycle have been identified and characterized, including a unique bis-μ-oxo (Fe^{IV})₂ (Q) species that directly hydroxylates hydrocarbon substrates. Transient kinetic and isotope effect studies have led to suggestion of a radical-based mechanism for the reaction. However, the use of radical clock reagents as diagnostic chemicals have failed to lead to a definitive conclusion because the observation of the characteristic molecular rearrangements depends on the structure of the probe used. As a result, radical, cation, and concerted mechanisms have all been proposed. All of the studies in which a radical intermediate was detected show that it has a very short lifetime, on the order of 20-100 ps [1,2], so that direct freeze quench trapping or trapping by formation of a spin-adduct with an external compound are unlikely to be successful. Here we report a novel approach to this problem through the use of a "Two-in-One" molecule in which one end functions as an MMO substrate while at the other end carries a nitroso group to act as a fast spin trap. When a radical is formed on the substrate end, a portion of the spin density migrates with almost no dead time to the spin trap end where it is stabilized and can be visualized by EPR. Nitrosobenzene has been found to be an ideal "Two-in-One" molecule for the MMO system. Kinetic studies shows that NADH is consumed when it is used as an MMO substrate under the standard assay conditions, and it inhibits the oxidation of other substrates such as nitrobenzene suggesting that it binds in the active site. Following turnover of nitrosobenzene, EPR at both room and low temperatures has revealed a spin-trapped organic radical with a half lifetime of ~70 min at 22 °C. The radical signal is absent if MMO is inactivated or if any of the protein components required to transfer electrons from NADH to the MMO hydroxylase component are not present. The kinetics and EPR data presented strongly suggest that the observed radical is substrate-based and that it is an intermediate occurring at the stage of the substrate hydroxylation in MMO catalyzed reaction.

[1] Yi Jin and John D. Lipscomb, *Biochem. Biophys. Acta*, 1543, 47-59, 2000.

[2] Brian J. Brazeau, Rachel N. Austin, Carly Tarr, John T. Groves, and John D. Lipscomb, *J. Am. Chem. Soc.*, 123, 11831-11837, 2001.